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Rapid and inexpensive NaOH based direct PCR for amplification of nuclear and organelle DNA from ramie (*Boehmeria nivea*), a bast fibre crop containing complex polysaccharides

Pratik Satya[a,*], Sabyasachi Mitra[a], D.P. Ray[b], B.S. Mahapatra[a], M. Karan[a], S. Jana[a], A.K. Sharma[a,b]

[a] Central Research Institute for Jute and Allied Fibres, Barrackpore, Kolkata, India
[b] National Institute of Research on Jute and Allied Fibre Technology, Kolkata, India

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ABSTRACT

Most of the PCR based approaches in plant science rely on lengthy and expensive DNA isolation protocols, which often involve use of hazardous chemicals. Direct PCR methods save time and cost of experiments and also increase efficiency of PCR. We have compared three rapid DNA extraction processes for direct PCR in ramie (*Boehmeria nivea*), a fibre crop species containing high amount of gummy complex polysaccharides and developed modified protocols for direct PCR using NaOH as extraction buffer and Tris/Tris–HCl/Tris–EDTA as dilution buffer. These protocols were successful in amplification of nuclear DNA from leaf and stem tissues using ISSR and SSR markers and also chloroplast DNA amplification using primers for *rbc*L gene. Our results also show that nature and quantity of dilution buffer are important for increasing efficiency of direct PCR. The NaOH based methods are simpler, cheaper and economical compared to other direct PCR methods and work very well for tissues containing high amount of complex polysaccharides. The protocols are suitable for batch processing and high throughput genotyping within a short time period, which will have many applications in plant genomic researches.

1. Introduction

Polymerase chain reaction (PCR) is a DNA amplification procedure routinely used in molecular biology and biotechnology experiments. Typically, the template DNA for PCR amplification is extracted from a source organism followed by targeted amplification of gene/DNA sequences using random or specifically designed primers (Mullis and Faloona, 1987). PCR is extensively used for DNA marker based studies, DNA sequencing, cloning and identification of transformants, DNA barcoding, mutant identification, forensic studies and many other biological studies. PCR based DNA markers have versatile applications in plants from genotype identification, purity testing, genetic map construction, gene discovery, QTL mapping and marker assisted crop improvement (Agarwal et al., 2008).

DNA extraction is an integral component of all the PCR based experiments. To improve the speed and efficiency of PCR, numerous protocols have been developed to reduce the time period and to improve the quality of extracted DNA, most of which are based on hexadecyltrimethylammonium bromide (CTAB) or sodium dodecyl sulfate (SDS), ionic detergents used to disrupt tissues for releasing DNA from cell (Doyle and Doyle, 1987; Goldenberger et al., 1995). DNA extraction based on these methods requires about 24–48 h before the DNA is ready for PCR (Bellstedt et al., 2010). A number of commercial kits are also available for plant DNA extraction, which are more efficient but are expensive.

To eliminate the time consuming DNA extraction step from PCR, direct PCR amplification from ground plant tissues has been proposed (Berthomieu and Meyer, 1991). By reducing the pre-PCR steps from days to hours, direct PCR methods allow large scale processing of samples and minimize costs involved in DNA extraction. In spite of these advantages, only a few direct PCR protocols have been developed to date for plant species because of low success rate due to low yield of DNA and presence of inhibiting compounds like sugars, proteins and phenolics in reaction mixture (Rogers and Parkes, 1999). Some of these protocols are single step PCR, avoiding any storage of the extract (Rogers and Parkes, 1999), while others involve two or more sequential steps involving extraction of the tissue in one buffer followed by PCR amplification of the aliquot in reaction mixture (Bellstedt et al., 2010; Flores et al., 2012), which permit intermediate storage of the extract for longer periods.

Ramie, rhea or China grass (*Boehmeria nivea* (L.) Gaud.) is a perennial fibre crop producing high quality bast fibre for textile applications. The history of ramie cultivation is at least 5000 years old in China and Indo-Malay peninsula (Kirby, 1963; Liang et al., 2009). It is also grown in Japan, Brazil, the Philippines, India and

* Corresponding author. Tel.: +91 3325350415.
E-mail address: pscrijaf@gmail.com (P. Satya).

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Table 1
List of primers used in the experiment.

SL No.	Primer name	Sequence (5′–3′)		Primer length (nt)	Annealing temperature (°C)	Amplicon size (bp)
1	SISSR 6		BDB(CAC)$_5$	18	56.0	280–720
2	SSR 634	F	GGAGAATATAAGGCCGCGTAG	21	51.0	650
		R	CAGCGGTGTAAGGCTCTCTC	20		
3	*rbc*L	F	ATGTCACCACAAACAGAAACTAAAGCAAGT	30	48.0	1380
		R	CTTCACAAGCAGCAGCTAGTTCAGGACTCC	30		

B – (C, G, T); D – (A, G, T).

Korea. The fibre produced from ramie is strongest of all known plant based fibres bearing more than twofold strength of cotton fibre with a very high fibre cell length/breadth ratio (>3500) (Sarkar et al., 2010). Besides, the fibre bears additional useful properties, such as resistance to bacterial degradation and higher tensile strength under hygroscopic condition. The root of the plant is also used for medicinal purpose as antioxidant, anti-inflammatory and hepatoprotective agent (Lin et al., 1998; Huang et al., 2009). The stem of ramie contains high amount of mucilaginous and complex pectinaceous substances, commonly described as gum. During processing, the gummy substances are removed chemically or enzymatically before fibre extraction (Bruhlmann et al., 1994). Presence of such complex polysaccharides reduces the efficiency of CTAB based DNA isolation methods and also may interfere with PCR (Kaufman et al., 1999). There are some reports of extraction of genomic DNA from cotyledons or young ramie leaves using CTAB based methods (Liang et al., 2009; Li et al., 2010), but no rapid method for direct PCR or quick DNA extraction from leaves or stem is available.

Here we describe methods for direct PCR from ramie stem and leaf tissues, using NaOH and Tris/Tris–HCl/Tris–EDTA. The NaOH based direct PCR method is modified from the alkali based quick DNA extraction method developed by Wang et al. (1993). Since these methods work well with high gum containing species like ramie, we believe it will be useful for direct PCR for many plant species.

2. Materials and methods

2.1. Plant material

Ramie (*B. nivea* L. Gaud) material was cv. R 67-94, grown at Central Research Institute for Jute and Allied Fibres, Kolkata, India. Leaf and stem samples were collected from 35 days old plant at active vegetative growth phase. Stem samples were collected from apical soft stem region as well as basal hard stem region. All samples were collected in polypropylene bags and stored at −20 °C prior to use.

2.2. Direct PCR

Three protocols for quick DNA extraction from plant tissues were first tested to develop a direct PCR amplification protocol in ramie using leaves, soft (upper) stem and hard (lower) stem tissues as described below. A total of four replicates (five samples per replicate) were tested for each method.

1. Rapid one step extraction method (ROSE) (Steiner et al., 1995) – Fresh tissues (50 mg) were ground in 500 μl extraction buffer (10 mM Tris–HCl, pH 8.0; 312.5 mM EDTA, pH 8.0; 1% PVP; 1% sodium lauryl sulphate, SDS) in a 2 ml Eppendorf tube, and incubated at 90 °C for 20 min, followed by freezing for 5 min. We used SDS instead of sodium lauryl sarcosinate, as it is more common anionic agent for DNA extraction (Goldenberger et al., 1995). 10 μl of the extract were diluted in 1690 μl of sterile H_2O. Five microlitre of the dilution was used as the DNA template for PCR.
2. Sucrose prep method (Berendzen et al., 2005) – DNA was extracted from fresh tissues (25 mg) on ice using 500 μl extraction buffer (50 mM Tris–HCl, pH 7.5; 300 mM NaCl, 300 mM sucrose), heated at 99 °C for 5 min and centrifuged at 5000 × *g* for 5 s. One microlitre of the dilution was used for PCR.
3. NaOH extraction method (Wang et al., 1993) – Tissue samples (30–50 mg) were ground in 300–500 μl of 0.5–1.0 N NaOH in a 2 ml Eppendorf tube with the help of a small plastic pestle for a few minutes (alternatively, tissue can be ground in a mortar by pestle and transferred to tube). The material was then spun at 5000 × *g* for 30 s using a standard low speed bench top centrifuge to precipitate the large particles. Five microlitre of the supernatant was then diluted to 1:100 ratio in 0.1 M Tris (pH 8), vortexed for a few seconds and further diluted to 1:10 ratio in 0.1 M Tris (pH 8). At this stage, the material can be stored at −20 °C or −80 °C for future use if required. One microlitre of the solution was used as PCR template either in a 0.2 ml PCR tube or in strips of 8 tubes using multichannel pipettes. We have modified the original method by varying the concentration of NaOH used, by introducing an additional step of centrifugation to avoid pipetting of larger tissue particles and also by optimizing the dilution of the aliquot. The protocol was further modified by replacing Tris by Tris–HCl (0.1 M) or Tris–EDTA (0.1 M) in the dilution buffer. We also tried to use 1:1 dilution of the NaOH extract in Tris (0.1 M) to develop a single step PCR method. Here, after grinding of tissue samples in 0.5 M NaOH, equal volume of 0.1 M Tris (pH 8.0) was added in the same Eppendorf tube and 1 μl of the solution was directly used as PCR template.

PCR amplifications were carried out using the following protocols. The final reaction volume was 20 μl containing 1 μl (NaOH and Sucrose Prep methods) or 5 μl (ROSE method) of template DNA, 2 μl of 10× PCR buffer, 0.4 μl of 10 pmol dNTPs (Invitrogen, USA), 0.4 μl of 25 mM $MgCl_2$, 0.8–1.2 μM of primers and 0.5 μl of 3 U Taq DNA polymerase (Invitrogen, USA). The amplification was carried out in a thermal cycler (Agilent, USA) with following programme: 4 min at 94 °C, 43 cycles of 1 min at 94 °C, 1 min at primer specific annealing temperature and 2 min at 72 °C followed by 8 min at 72 °C for final extension.

Three different marker systems, nuclear simple sequence repeat (SSR) from jute (*Corchorus olitorius*) (SSR 634), inter simple sequence repeat (ISSR) from potato (*Solanum tuberosum*) (SISSR 6) and primers for the chloroplast *rbc*L gene sequence were tested for direct PCR amplification (Table 1). Amplified products using ISSR and SSR primers were resolved at 1% agarose gel, while for chloroplast primer, 1.5% agarose gel was used. Amplified products were visualized and photographed using a gel documentation system (Biorad, USA). The intensity of the amplicons was compared using gel image analysis software (Quantity one Ver.4.6.3, Biorad, USA).

3. Results

Out of ROSE, sucrose-prep and NaOH based DNA extraction methods, only the NaOH based direct PCR method yielded